HISTOLOGY AND METABOLISM OF FROZEN INTERTIDAL ANIMALS[1]

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Many invertebrate animals are normally exposed to environmental temperatures far below the freezing point of their body fluids. Although supercooling may sometimes be a factor in survival (Salt, 1950; Ditman *et al.*, 1942; Scholander *et al.*, 1953), freezing occurs in nature among insects (Asahina *et al.*, 1954; Scholander *et al.*, 1953), shore animals (Kanwisher, 1955), and other groups (Luyet and Gehenio, 1940.) On the shore during winter, for example, freezing and thawing occurs twice a day when the animals are exposed to the cold air by the tide. Intertidal animals in the Arctic may be frozen for as long as 6 months (Kanwisher, 1955). The survival of these animals depends on their being able to have most of their body water turned to ice. It is remarkable that no injury is produced in a living system when more than half of its bulk is changed to a crystalline solid. I am reporting here some investigations on the histology and metabolism of these intertidal animals.

Histology

In the freeze-drying histological technique, tissue is cooled very quickly with liquid nitrogen. Freezing occurs so fast that ice crystals do not have time to grow very large and cellular organization is very little disturbed. The water is removed by vacuum while the sample is kept cold. The resulting dehydrated tissue matrix is imbedded, sectioned, and stained in a conventional manner. I have used the method here to capture the situation in tissue from shore animals frozen to relatively mild natural temperatures. Comparison with material from unfrozen animals has shown the distortions caused by the freezing.

Animals were collected from the shore at Woods Hole in January and moved to a $-10°$ cold room without thawing. Sections of tissue about 1 mm. thick were cut with a cold knife, held with cold tweezers, and plunged into a vial of isopentane suspended in a container of liquid nitrogen. The isopentane allows a faster heat transfer because it does not boil and form an insulating gas layer. The hard frozen samples were quickly transferred to the already cold dehydrating chamber and vacuum applied for 24 hours at about $-45°$. The dehydrated tissue was then imbedded in de-gassed paraffin already in the chamber with the vacuum still applied. Photomicrographs of 10-micron sections are shown in Figure 1. The unfrozen controls were tissue taken from identical animals that had thawed at room temperature for an hour.

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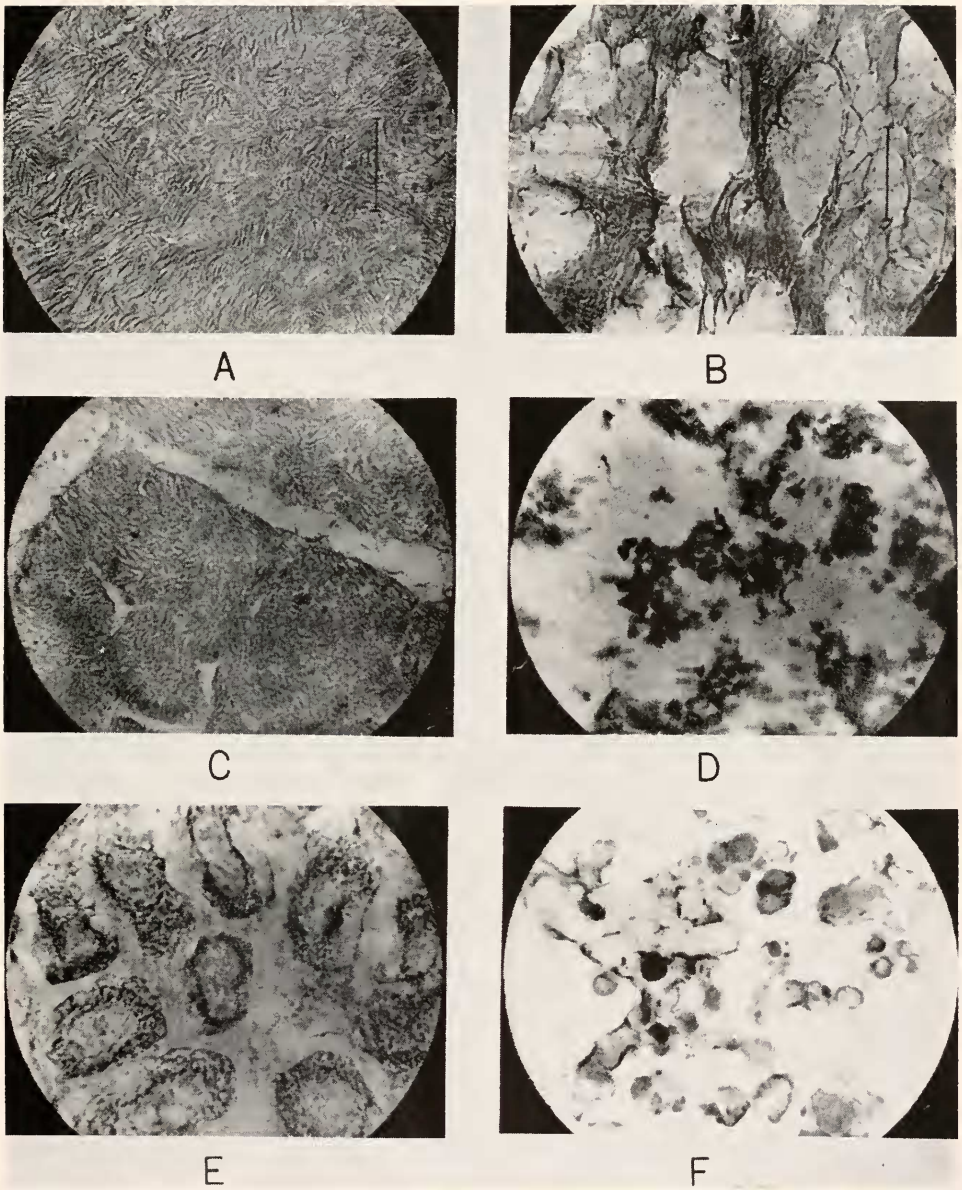

FIGURE 1. Photomicrographs of unfrozen and frozen tissue.

Figure 1, A is the unfrozen foot of the shore snail *Littorina littorea*. The purpose of the randomly arranged muscle fibers is related to the snail's type of locomotion. In the frozen tissue in Figure 1, B, the ice forms in large pockets with a resulting shrinkage and distortion of the cells.

The extreme distortions indicated in the initial results were surprising enough

to warrant the following procedure. The frozen muscle slice was cut in two pieces. One was used as the frozen specimen. The other was warmed for less than a minute on the palm of my hand and then hard frozen in the liquid nitrogen. When sectioned it appeared nearly the same as tissue from an unfrozen animal. Figures 1, A and 1, B are actually sections from this run.

A transverse section of the unfrozen adductor muscle in the oyster, *Crassostrea virginicus,* is shown in Figure 1, C. The parallel muscle fibers are viewed end-on. In its frozen counterpart in Figure 1, D, the fibers are clumped into groups to make room for the intervening ice. The prominent elements that resulted are all about the same size. There may be membranes not visible in the unfrozen muscle to account for this regularity. The same regular clumping was seen in the adductor muscle of two mussels, *Modiolus modiolus* and *Mytilus edulis.*

Figure 1, E is of the eggs in the unfrozen ovary of the blue mussel *Mytilus edulis.* When frozen as in Figure 1, F, the detail is much less distinct but the eggs clearly have shrunken during the formation of the large amounts of intercellular ice. Comparable distortions were seen in other tissues from these and other species.

Metabolism of Frozen Animals

Scholander *et al.* (1953) measured respiration at freezing temperatures by following the decrease in oxygen concentration in a closed volume containing the animal. The same method has been used here. Manometric and volumetric techniques can not be used because of the volume change when water turns to ice.

The snails to be used were frozen in 20-ml. syringes in a cold bath. Only those in which the snail froze while fully extended from its shell were used. A short section of tubing on the tip of the syringe extended above the surface of the liquid and was closed with a pinch clamp. A sample of gas could be withdrawn without removing the syringe from the bath. The plunger was free to move up and replace the volume lost in sampling. Allowance was made for the decreased volume in calculating the rate of oxygen removal.

Duplicate oxygen analyses good to 0.02 per cent were made with the half-cc. analyzer of Scholander (1947). Serial samples were plotted against time and the slope was used in computing the oxygen consumption. The concentration was never allowed to go below 18 per cent in any run. Respiration was assumed to be independent of tension over this small range.

After the snails were placed in the cold bath, at least 6 hours were allowed for phase equilibration between ice and water in the tissues. Previous experience (Kanwisher, 1955) had shown that there was no appreciable increase in ice after this length of time. The syringe was then flushed with cold outside air. A series of oxygen determinations showed that such air did not vary appreciably from 20.94 per cent so this was considered the starting concentration. At intervals ranging from 2 to 120 hours samples were withdrawn with a mercury gas sampler.

Volumetric respirometers (Scholander *et al.*, 1952) were used above 0°. One ml. of sea water was included in the vial with the animals. At 0° such values were in good agreement with those made by gas analysis which is specific for oxygen. The often used and rarely proven hypothesis is thus confirmed that the volume decrease is due to oxygen being consumed.

The respiration temperature data from −10 to +30° C. are plotted in Figure 2. Between 0 and +20°, oxygen consumption shows the usual logarithmic increase

with a Q_{10} of 2 to 3. Above this respiration decreases, probably due to thermal injury. Below 0° the metabolic activity drops sharply with an apparent Q_{10} of about 50.

At −10°, respiration was so low it took 6 days for the snails to consume a measureable amount of oxygen. Even in this length of time the concentration change was smaller than desired for accurate determination. This may account for the greater spread of values at this low temperature. At −15° the empty syringes gave blank values of one-third the oxygen decrease in those containing snails. This may be due to oxidation of grease used on the syringe plunger. It

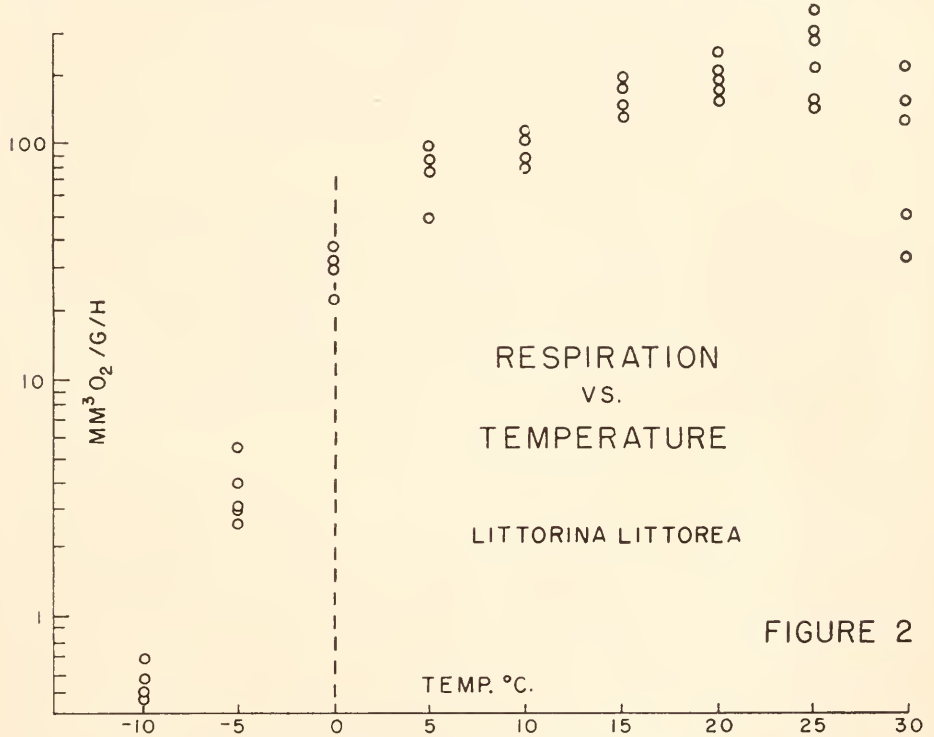

FIGURE 2. Variation of oxygen uptake with temperature.

did not seem that this technique could be trusted on the slower rates to be expected at still lower temperatures.

SALINITY EFFECT ON RESPIRATION

Scholander *et al.* (1953) have given several reasons why the respiratory gas exchange of a frozen animal drops so much more rapidly with temperature than it does above 0° when no ice is present. The ice may act as a diffusion barrier to the gases. The increased viscosity of the body fluids may slow the reaction rates. Finally the increased salinity may directly inhibit the animal's metabolism. No way could be devised to test the first two hypotheses. The respiratory response to increased salinity above 0° can be determined independently of any ice effects.

Higher than normal salinities were made by freezing sea water and using the brine. Dilution with fresh water gave lower than normal salinities. Freshly collected snails were placed in jars containing the different salinities for a minimum of 6 hours before being used. At very high and low values the snails withdrew into their shells. Experience had shown that the operculum blocks respiratory gas exchange so these could not be used.

For the respiration measurements single snails were placed in 20-ml. syringes filled with the desired salinity. The syringes were kept in a constant temperature bath except when sampling. One-ml. samples were removed at convenient intervals and analyzed gasometrically for oxygen by the method of Scholander *et al.* (1955).

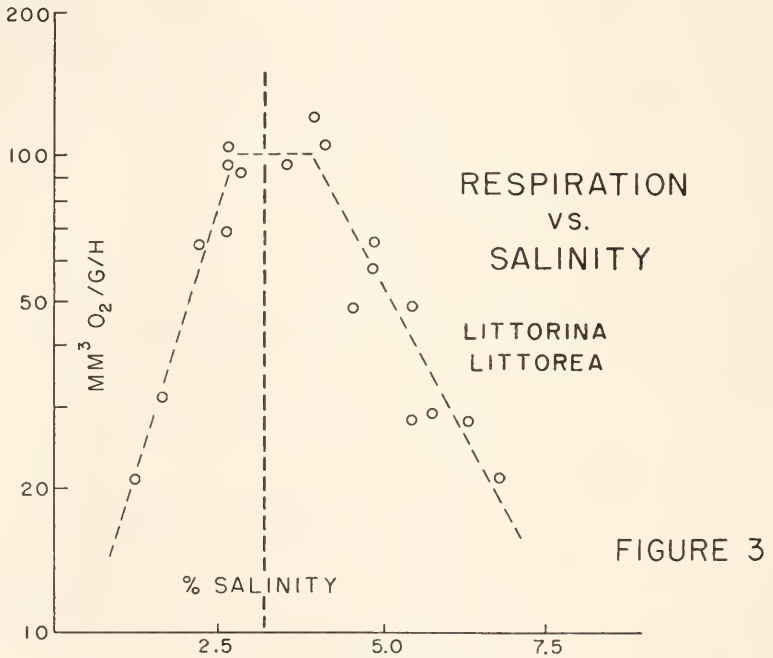

FIGURE 3. Response of oxygen uptake to different salinities.

Since this is a physical extraction of the gases it could be relied on in spite of the water sometimes becoming cloudy with waste products.

As in the low temperature gas analysis method several serial readings were used to indicate the rate of oxygen removal by the snails. Low oxygen tensions were avoided by working in the range of 2.5 to 6 mm.³ of oxygen per ml. The curves showed that respiration was independent of tension over this range.

The variations of oxygen consumption with changes in the external salinity are shown in Figure 3. High salinity depresses the respiration of *Littorina littorea*. This is a reversible effect since the rate increases again when the snail is returned to normal salinity. When the snails withdrew into their shells at higher salinities than shown, no oxygen consumption could be detected. They are apparently able to subsist for long periods anaerobically.

Since freezing occurs throughout the animal, the remaining body fluids in all parts of the animal are concentrated. If any effect of external salinity above 0° is to be related to freezing, it must be shown that the animal is not osmotically regulating. Increased salinity could conceivably be effective in only altering the absorption of oxygen at the surface. Tissues of snails from water of different salinities were analyzed for chlorides. By carefully cracking the shell, the animal could be removed whole. Excess water was mopped off and the weight quickly taken. The water was removed by drying for several hours in a 100° oven. The dry weight then gave the total water by difference. The dry tissue was then digested and titrated for the amount of total chlorides present. This amount was considered dispersed in all the animal water in order to calculate the concentration in the live animal. From the resulting normalities at different external salinities it was clear that internal chloride concentration was proportional to that outside the animal. Any result of externally varied salinity can reasonably be viewed as arising from a corresponding change throughout the animal.

Discussion

Chambers and Hale (1932) observed plant and animal cells freezing under the microscope. They found that ice formation inside the cellular membrane always resulted in the death of the cell. The detailed studies of Asahina and his colleagues (1954) have described the freezing process in the blood and isolated organs of insect pre-pupae and in the intact insects themselves. They also found that intra-cellular freezing is lethal to the cell or tissue. Such results are responsible for the general belief that all freezing in animals and plants takes place outside the cells.

The impressive liquid air freezing experiments of nematodes by Luyet and Gehenio (1940) have almost certainly been an instance of ice within cells. Lack of injury here has been attributed to the very rapid rate of freezing. This vitrification does not allow time enough for ice crystals to grow to a size where they can damage protoplasmic structures. It forms the basis of the histology used here. This phenomenon probably has little to do with the normal ecology of these animals in nature.

Shore animals that are exposed to freezing are in shells. This impedes heat transfer and gives ice crystals time to grow. One can readily see them in an opened animal. The tissues of such an animal are similar in texture and appearance to a frozen piece of meat. It is not surprising when one considers that water makes up three-fourths of the bulk of the animal and four-fifths or more of it may be ice. The photomicrographs presented here show the large amount of distortion necessary at the cellular scale to make room for this ice. Yet this can change back to a more or less normal appearing tissue in 30 seconds as shown in Figures 1, A and B. Siminovitch and Briggs (1949) have related frost hardiness in plants to the ability of water to migrate rapidly in and out of the cells. Unfortunately no equivalent test could be devised to apply this to shore animals.

Littorina littorea was found to remain out of its shell and behave normally in salinities of 2 to 7 per cent. It sharply decreased its metabolism in response to a salinity increase. In Figure 3, the data indicate that doubling the salinity above the optimum decreases the oxygen demand to about a third. As the salinity of

the body fluids is increased by the freezing out of water, oxygen uptake must drop in the same fashion. From the freezing curves in a previous paper (Kanwisher, 1955) 70 to 80 per cent of the water in this species is frozen at −10°. This would result in a Q_{10} from the salinity of about 10. Above 0° the Q_{10} due to the usual temperature effect on reaction rates is between 2 and 3. Combining these one would expect a Q_{10} in the range of 20 to 30 below 0°. The actually observed one is closer to 50. The effect of ice as a gaseous diffusion barrier and that from the loss of water itself may account for the difference. It is felt that the present data do not warrant a more vigorous interpretation. Similar work with intertidal algae (Kanwisher, 1957) has shown that the drying effect of freezing was chiefly responsible for a similar large decrease in respiration. A three-times increase in salinity had little effect on the oxygen uptake of these plants.

Freezing in shore animals to the extent shown here is a normal occurrence twice daily in the winter with no obvious injury to the animal. This freezing hardiness is probably connected with the ability to stand the internal distortions and high salinities that result. The greatly lowered metabolism may be of adaptive significance in severe locations where shore animals are frozen into the ice for months at a time. As such it could represent a considerable saving in food reserves.

Summary

1. Histology of frozen shore animals has shown large pockets of intercellular ice with consequent shrinkage and distortion of the surrounding cells.

2. The Q_{10} drops precipitously in the region of ice formation and may be as high as 50.

3. High tissue salinity without freezing decreases oxygen uptake. Thus the salinity increase that results from freezing is responsible for a large part of the high Q_{10}.

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